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Immunological monitoring of extracorporeal photopheresis after heart transplantation

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Summary

Extracorporeal photopheresis (ECP) has been used as a prophylactic and therapeutic option to avoid and treat rejection after heart transplantation (HTx). Tolerance-inducing effects of ECP such as up-regulation of regulatory T cells (T_{regs}) are known, but specific effects of ECP on regulatory T cell (T_{reg}) subsets and dendritic cells (DCs) are lacking. We analysed different subsets of T_{regs} and DCs as well as the immune balance status during ECP treatment after HTx. Blood samples were collected from HTx patients treated with ECP for prophylaxis ($n = 9$) or from patients with histologically proven acute cellular rejection (ACR) of grade ≥ 1B ($n = 9$), as well as from control HTx patients without ECP (HTxC; $n = 7$). Subsets of T_{regs} and DCs as well as different cytokine levels were analysed. Almost 80% of the HTx patients showed an effect to ECP treatment with an increase of T_{regs} and plasmacytoid DCs (pDCs). The percentage of pDCs before ECP treatment was significantly higher in patients with no ECP effect (26·3% ± 5·6%) compared to patients who showed an effect to ECP (9·8% ± 10·2%; $P = 0·011$). Analysis of functional subsets of $CD4^+CD25^{high}CD127^{low}$ T_{regs} showed that CD62L-, CD120b- and CD147-positive T_{regs} did not differ between the groups. CD39-positive T_{regs} increased during ECP treatment compared to HTxC. ECP-treated patients showed higher levels for T helper type 1 (Th1), Th2 and Th17 cytokines. Cytokine levels were higher in HTx patients with rejection before ECP treatment compared to patients with prophylactic ECP treatment. We recommend a monitoring strategy that includes the quantification and analysis of T_{regs}, pDCs and the immune balance status before and up to 12 months after starting ECP.

Keywords: dendritic cells, extracorporeal photopheresis, heart transplantation, immune balance, regulatory T cells

Introduction

Extracorporeal photopheresis (ECP) has been reported to be effective to reverse acute rejection after solid organ transplantation [1]. The successful application of ECP was demonstrated after kidney, heart, lung, liver, pancreas and bone marrow transplantation [2]. Several studies have proven the effectiveness of ECP in combination with triple-drug immunosuppressive therapy to decrease the risk of cardiac rejection [3,4]. Additionally, the applicability of ECP has been demonstrated as a prophylactic procedure to avoid rejection after heart transplantation (HTx) [5–7].

During ECP the patient's peripheral blood mononuclear cells (PBMC) become incubated with a photosensitive drug, which binds to the deoxyribonucleic acid (DNA) of the PBMCs after it has been activated by ultraviolet-A light. This irradiation causes structural damage to the DNA, resulting in apoptosis of the PBMCs which return to the patient's circulation.

ECP treatment induces immune regulatory processes that manifest clinically in an increased tolerance of the transplanted organs. Regulatory T cells (T_{regs}) are a subpopulation of T cells that are involved in immune modulatory processes and in the maintenance of self-tolerance [8,9]. T_{regs} might act multi-directionally by preventing migration of effector T cells, natural killer cells or B cells to target organs, resulting in their inability to co-operate with antigen-presenting cells, thus inducing

anergy [10]. Subsets of T_{regs} can be identified by surface markers, which have been described by different groups [11–16], and allow the functional characterization of T_{regs}; for example, the surface markers CD39 and CD120b have been detected on T_{regs} with a high suppressive potential [11,12]. Activated memory-like T_{regs} (T_{REM}) express high levels of CD62L, CD27 and CD39 [12,15,17]. Another surface marker, CD147, divides $CD4^+CD25^+$ cells into distinct subsets and marks the switch between resting and activated subsets of forkhead box protein 3 ($FoxP3^+$) T_{regs} [13].

As well as T_{regs}, dendritic cells (DCs) play a central role in the induction of immune responses and in the maintenance of self-tolerance [18]. For the different DC subsets, plasmacytoid (pDCs) and myeloid (mDCs) dendritic cells, it has been shown that no significant changes in the DC populations occurred over a 12-month period after initiation of ECP in patients with chronic graft-*versus*-host disease [19].

Only a few studies that describe ECP treatment include monitoring data of immunological markers. For the application of ECP after HTx the data are missing. The monitoring of immune modulatory effects caused by ECP treatment is of great clinical relevance because, to date, no detailed diagnostic strategy is available to evaluate the individual success of this intervention.

In our study, we analysed different subsets of T_{regs}, mDCs and pDCs as well as the immune balance status during ECP treatment in HTx patients. In this way, it may be possible to acquire further evidence of detailed ECP-induced immunological mechanisms. According to our data, we developed a monitoring strategy for ECP treatment after HTx.

Methods

Patient population and sample collection

This study was approved by the Ethics Committee of the Medical Faculty of the University Leipzig, and all patients gave written informed consent. The study population comprised 25 HTx patients and was divided into three study groups. HTx patients without biopsy-proven acute cellular rejection in the first 3 months post-transplant were included in the 'prophylactic ECP group' ($n = 9$), while we included HTx patients with a biopsy-proven acute cellular rejection at study start in the 'acute cellular rejection (ACR) study group' ($n = 9$). In our control study group we included clinically stable long-term HTx patients without biopsy-proven acute cellular rejection 1 year before the study began and without ECP therapy ($n = 7$). Heparinized whole blood samples and sera were collected before and after ECP treatment and of HTx patients of the control group at their out-patient visit. Whole blood was used directly for flow cytometric analysis. Sera were aliquoted and stored at −80°C until multiplex screenings.

ECP treatment of HTx patients

One ECP cycle consisted of ECP treatments on 2 consecutive days with the Therakos™ CELLLEx™ Photopheresis System and UVADEX™ drug (both Therakos Inc., Raritan, NJ, USA). ECP treatment was not undertaken in the case of infectious disease in HTx patients. Two different indications required ECP treatment: (i) prophylactic treatment in combination with calcineurin-inhibitor-free immunosuppression in the first year post-HTx and (ii) biopsy-proven acute cellular rejection [grade IB or higher; International Society of Heart and Lung Transplantation (ISHLT), 1990]. The ECP treatment included two ECP sessions on 2 consecutive days every 4 weeks during a period of 3 months. Heparinized whole blood samples and sera were collected before each ECP treatment.

Flow cytometric analysis

Flow cytometric data were acquired and analysed using BD™ LSR II Flow Cytometer [Becton Dickinson (BD), Heidelberg, Germany] and BD FACSDiva version 6·1·3 software. Peripheral blood dendritic cell subsets were quantified as described previously [20]. T_{regs} were defined as $CD3^+$ $CD4^+$ cells that showed high expression of CD25 ($CD25^{high}$) and low expression of CD127 ($CD127^{low}$). Whole populations of $CD3^+CD4^+CD25^{high}CD127^{low}$ T_{regs} and functional subsets of T_{regs} were quantified by means of two different antibody panels [panel 1: CD3-peridinin chlorophyll-cyanin 5·5 (PerCP-Cy5·5), CD4-antigen-presenting cells (APC)-H7, CD25-phycoerythrin (PE)Cy7, CD127-APC, CD120b-PE, CD147-fluorescein isothiocyanate (FITC); panel 2: CD3-PerCP-Cy5·5, CD4-APC-H7, CD25-PECy7, CD127-APC, CD39-FITC, CD62L-PE]. All antibodies were obtained from BD, except CD127-APC (Biolegend, Fell, Germany). Antibody mixture from panels 1 or 2 was incubated with 200 µl of human whole blood for 20 min at room temperature (RT) in the dark. Fluorescence-activated cell sorter (FACS) lysing solution (BD) was added, and samples were incubated for 10 min. After centrifugation at 300 ×***g*** for 5 min, supernatant was discarded and samples were washed with 4 ml phosphate-buffered saline (PBS) (pH 7·4). Supernatant was discarded, samples were mixed well and 500 µl 1% formaldehyde-PBS solution was added. Samples were stored until analysis at 4°C. Samples that were stained with antibodies against CD3, CD4, CD25 and CD127 served as staining-negative controls; 10 000 events of $CD3^+CD4^+$ cells were analysed per sample.

Quantification of cytokines

Cytokine quantification for assessment of immune balance was performed using a Milliplex MAP Human Cytokine/

Table 1. Overview about the demographic data, immunological risk, immunosuppressive regimen and the endomyocardial biopsy grading up to 6 months after starting extracorporeal photopheresis (ECP) of heart transplantation (HTx) recipients with and without ECP treatment.

	ECP prophylaxis group ($n = 9$)	ECP ACR group ($n = 9$)	HTx control group ($n = 7$)	*P*-values
Demographic data				
Age at study begin	53·7 ± 12·8 years	55·0 ± 8·7 years	60·3 ± 8·6 years	0·475[a]
Gender % male	67% ($n = 6$)	89% ($n = 8$)	86% ($n = 6$)	0·452[b]
Patients with immunological risk				
Retransplantation	0	0	0	1[c]
PRA < 5% (before HTx)	100% ($n = 9$)	100% ($n = 9$)	100% ($n = 7$)	1[c]
Time between HTx and study beginning	3·0 ± 0 months	14·6 ± 10·1 months	135·4 ± 39·3 months	<0·05[a]
Immunosuppressive regimen at study beginning[§]				
Cyclosporin A, everolimus	56% ($n = 5$)	56% ($n = 5$)	0% ($n = 0$)	0·039[b]
Everolimus, mycophenolic acid	44% ($n = 4$)	22% ($n = 2$)	29% ($n = 2$)	0·585[b]
Tacrolimus, mycophenolic acid	0% ($n = 0$)	0% ($n = 0$)	29% ($n = 2$)	0·145[b]
Cyclosporin A, mycophenolic acid	0% ($n = 0$)	22% ($n = 2$)	29% ($n = 2$)	0·396[b]
Everolimus, tacrolimus	0% ($n = 0$)	0% ($n = 0$)	14% ($n = 1$)	0·396[b]
EMB grading (ISHLT) 3 months after starting ECP				
Grade IA	78% ($n = 7$)	89% ($n = 8$)	–	1[c]
Grade IB	22% ($n = 2$)	11% ($n = 1$)	–	1[c]

[a]*P*-value determined by one-way analysis of variance (ANOVA). [b]*P*-value determined by Pearson's χ^2 test. [c]*P*-value determined by Fisher's exact *t*-test. [§]In combination with glucocorticoids. ACR = acute cellular rejection; EMB = endomyocardial biopsy; ISHLT = International Society of Heart and Lung Transplantation; PRA = panel of reactive antibodies.

Chemokine Panel (MerckMillipore, Darmstadt, Germany) and the Luminex 100 IS System (Luminex, Austin, TX, USA) with Luminex 100 IS 2·3 software. Interleukin (IL)-2, IL-4, IL-10, IL-17 and interferon (IFN)-γ were quantified with a standard curve ranging from 3·2 to 10 000 pg/ml, according to the manufacturer's instructions. For assessment of immune balance, cumulative values for the T helper type 1 (Th1) cytokines (IL-2, IFN-γ) and Th2 cytokines (IL-4, IL-10), as well as the ratio between these two values (Th1/Th2 ratio), were calculated.

Classification of ECP-treated patients

We used the cellular parameters 'percentage of T_{regs}' on $CD3^+CD4^+$ cells and 'percentage of pDCs' on the whole DC population to classify HTx recipients into 'positive effect' and 'no effect' of ECP therapy as follows: (i) 'positive ECP effect' were HTx recipients with a minimum of 20% increase of percentages of T_{regs} or pDCs within the first two completed ECP cycles (including the period until the following ECP cycle was started); and (ii) 'no ECP effect' were HTx recipients with a less than 20% increase of percentages of T_{regs} or pDCs within the first two completed ECP cycles.

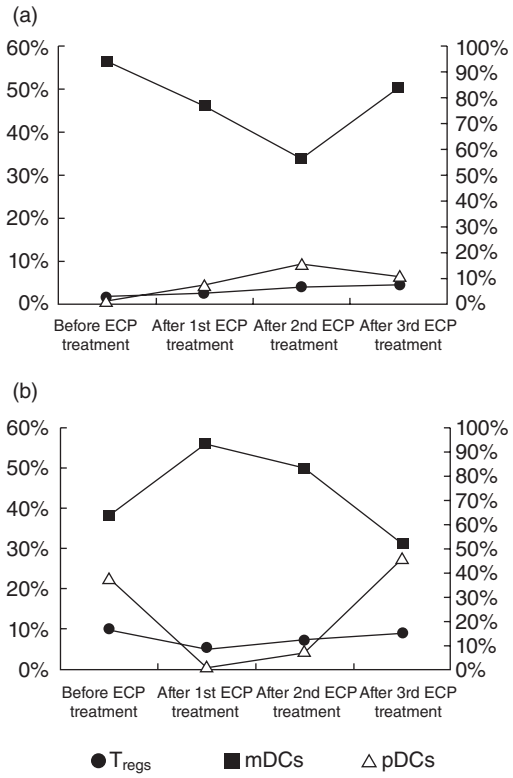
Statistical analysis

Statistical analysis was performed using SPSS statistical software version 17·0 (IBM Corporation, Armonk, NY, USA). Unless stated otherwise, data are displayed as mean ± standard deviation. For all analyses, *P*-values <0.05 were considered statistically significant. Means were compared with the unpaired Student's *t*-test when comparing two groups, or with one-way analysis of variance (ANOVA) for analysis of more than two groups. If equality of variances differed significantly between the groups, the Welch test was interpreted. The immunological response to the ECP treatment was analysed using the data sets of cellular parameters that were measured after two completed ECP cycles. Pre-ECP/post-ECP comparison of cellular parameters was performed using a general linear model (GLM) with repeated measurements.

Results

Identification of different profiles of cellular parameters

Regarding age, gender and immunological risk factors, such as retransplantation and panel of reactive antibodies (PRA) levels before transplantation, no differences were observed between patients with prophylactic ECP treatment, patients with ECP treatment after ACR and the HTx control group (Table 1). The duration between HTx and the start of the study differed between the groups ($P < 0·05$), just as the immunosuppressive combination therapy of cyclosporin A and everolimus differed at the start of the study ($P = 0·039$). Eight of nine patients (89%) in the ACR group responded to three ECP treatments, as the histopathological evidence of the first endomyocardial biopsies after three ECP treatments did not show a rejection grade higher than 1A (ISHLT, 1990). One patient in the ACR group showed no change in the histopathological grading of endomyocardial biopsies before and 3 months after starting ECP (Table 1).



	T_{regs} [%]	pDCs [%]	mDCs [%]
Before ECP	1·6%	0·9%	94·3%
After 1st ECP	2·7%	4·7%	77·1%
After 2nd ECP	3·9%	9·5%	56·5%
After 3rd ECP	4·7%	6·7%	84·5%

	T_{regs} [%]	pDCs [%]	mDCs [%]
Before ECP	9·9%	23·0%	63·4%
After 1st ECP	4·9%	0·2%	93·1%
After 2nd ECP	7·2%	3·9%	83·5%
After 3rd ECP	9·1%	27·8%	51·7%

Fig. 1. One patient is shown as an example for a positive extracorporeal photopheresis (ECP) effect (a) and one patient as an example without ECP effect (b) on the basis of percentage of T_{regs} on whole $CD3^+CD4^+$ T cells and percentages of plasmacytoid dendritic cells (pDCs) and myeloid DCs (mDCs) on the whole DC population. In contrast to patients without effect to ECP, patients with positive ECP effect showed distinct increases of regulatory T cells (T_{regs}) and/or pDCs not later than after the second completed ECP cycle. Left *y*-axes display the percentages of pDCs/DCs or $T_{regs}/CD3^+CD4^+$ cells. Right *y*-axes display the percentage of mDCs/DCs.

Two different profiles in the course of the cellular parameters within the first three ECP cycles could be identified (Fig. 1). Six of nine (67%) HTx patients with prophylactic ECP treatment had a positive ECP effect on T_{regs} or pDCs. Patients with ECP treatment after ACR had a positive ECP effect in all cases with an increase of T_{regs} or pDCs. While the percentages of T_{regs} and mDCs before ECP treatment did not differ in patients with positive ECP effect compared to patients with no ECP effect (T_{regs}: 5·9% ± 3·0% positive ECP effect *versus* 7·4% ± 2·6% no ECP effect, $P = 0{\cdot}427$; mDCs: 64·1% ± 18·7% positive ECP effect *versus* 64·0% ± 6·4% no ECP effect, $P = 0{\cdot}990$), the percentage of pDCs before ECP treatment was significantly higher in patients with no ECP effect (26·3% ± 5·6%) compared to patients with positive ECP effect (9·8% ± 10·2%; $P = 0{\cdot}011$). The percentage of pDCs in the HTx control group was 23·4% ± 3·7% before ECP treatment. Additionally, we detected that the percentage of pDCs before ECP treatment of patients with no ECP effect decreased dramatically during ECP treatment, but returned to or exceeded the initial level of pDCs during the further treatment process.

Analysis of the immunological response to ECP treatment

Statistical analysis of T_{regs} and DC subsets in HTx patients that were treated with ECP did not show statistical significances compared to untreated HTx recipients. Even if the indication for the ECP treatment is included in the statistics, no significant differences were detected because of the high interindividual variance (Fig. 2). However, slight increases were detected for T_{regs} of ECP-treated patients (5·8% ± 1·5%; median: 6·3% for the prophylaxis group; 6·1% ± 1·6%; median: 6·4% for the ACR group) compared to HTx patients without ECP treatment (5·2% ± 1·5%; median: 4·8%) (Fig. 2). The percentage of pDCs was reduced during ECP treatment (15·9% ± 6·5% for the ACR group; 21·6% ± 15·7% for the prophylaxis group) compared to the HTx control group (23·4% ± 10·7%). Furthermore, the quantified ECP treatment effect showed an average increase of T_{regs} by the factor 1·21 ± 0·55 (prophylaxis group: 1·21 ± 0·56; ACR group: 1·21 ± 0·55) and of pDCs by the factor 9·46 ± 13·31 (prophylaxis group: 10·26 ± 12·14; ACR group: 8·93 ± 14·00) within the first two completed ECP cycles.

Analysis of the functional subsets of $CD4^+CD25^{high}$ $CD127^{low}$ T_{regs} showed that the percentages of $CD147^+$, $CD120b^+$ and $CD62L^+$ T_{regs} did not differ between the ECP-treated groups and the HTx control group (Fig. 3). $CD39^+$ T_{regs} showed a similar profile to the whole T_{reg} population during ECP treatment: slightly increased, but non-significant, levels were detected for the ECP-treated groups (ACR group: 42·7% ± 22·3%, median: 48·0%; prophylaxis group: 33·9% ± 14·1%, median: 35·0%) compared to the HTx control group (28·0% ± 19·9%, median: 22·5%) (Fig. 3).

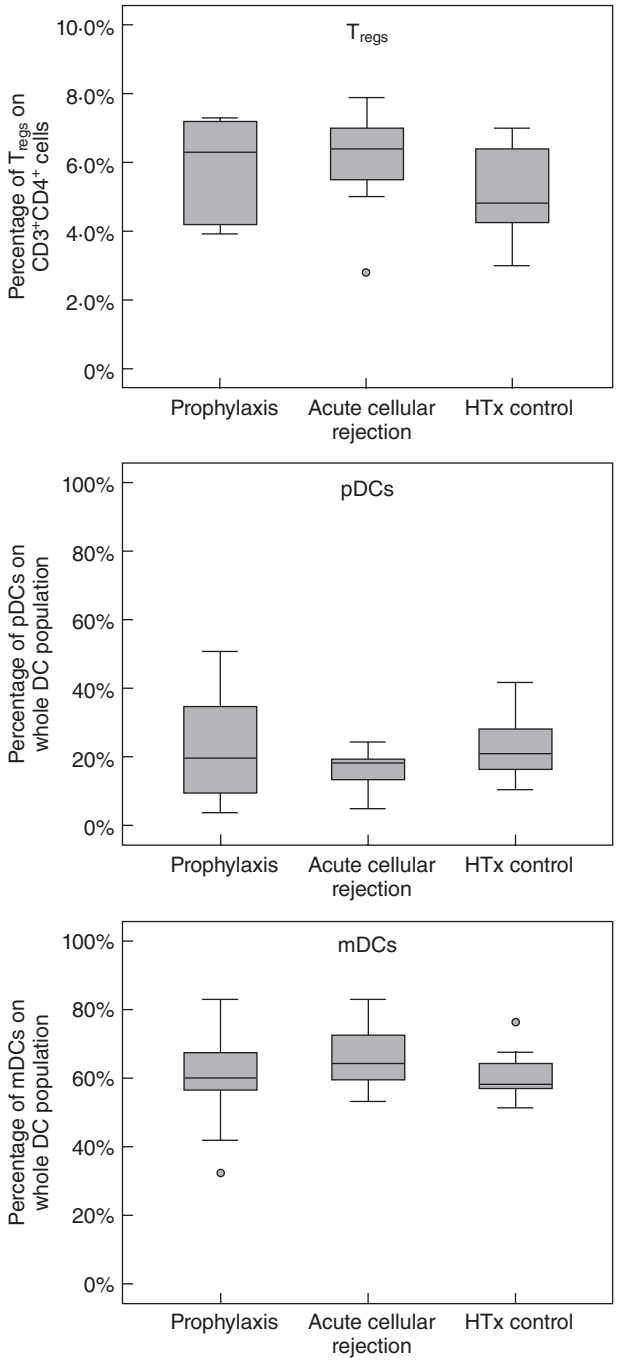


Fig. 2. Comparison of the T cell (T_{reg}) population and dendritic cell subsets in heart transplantation (HTx) patients with prophylaxis or rejection as indication for extracorporeal photopheresis (ECP) and in HTx patients without ECP treatment. Percentage of $CD25^{high}CD127^{low}$ T_{regs} on $CD3^+CD4^+$ T cells and percentages of plasmacytoid dendritic cells (pDCs) and myeloid DCs (mDCs) on the whole dendritic cell population are shown.

Effects of single ECP cycles on cellular and cytokine parameters

For identification of bias on interpretation of the cellular and cytokine parameters that originate from the ECP procedure and which might influence the effects of immunomodulation, we compared cellular parameters before and directly after one complete ECP cycle. Except for mDCs, cellular and cytokine parameters (T_{regs}, pDCs, IL-2, IL-4, IL-10, IL-17, IFN-γ) did not change directly after the ECP procedure. The percentage of mDCs changed significantly, from 71·3% ± 17·5% before to 60·6% ± 20·4% after the ECP cycle ($P = 0{\cdot}032$).

Analysis of immune balance during ECP treatment

The immune balance status was analysed in ECP-treated patients within the first two completed ECP cycles and in the HTx control group. The cytokine levels in the sera of all patients from the HTx control group were under the limit of detection. Therefore, we reasoned that these patients possessed a balanced immunological status. ECP-treated patients differed from the untreated control group and showed increased levels for Th1, Th2 and Th17 cytokines. Within the ECP-treated patients, differences were observed between the prophylactic-treated patients and the patients treated with ACR (Table 2): a shift towards increased Th2 cytokine levels were observed for the prophylactic ECP treatment group, while the ACR group showed the tendency for increased Th1 cytokine levels during ECP treatment. Furthermore, increased Th17 levels were observed for 22% ($n = 2$) of the patients from the prophylactic treatment group and for 56% ($n = 5$) of the patients from the ACR group.

Increased cytokine levels before ECP treatment were detected for one patient (11%) from the rejection group and for four patients (44%) from the prophylaxis group. All these patients with increased cytokine levels before the intervention showed a reduction of cytokine levels after the second complete ECP cycle. In some cases, cytokine levels increased again after additional ECP cycles (Fig. 4).

Discussion

To evaluate the efficiency of ECP treatment after HTx, we defined criteria for the classification of ECP-treated patients into 'positive ECP effect' and 'no ECP effect' according to the profile of cellular parameters. A comparable classification for ECP monitoring has not yet been reported, but considerable differences regarding the profile of quantified cell populations suggested a possibility for this classification. Furthermore, the classification is an ideal tool for assessment of the induction of immune modulatory processes during ECP treatment.

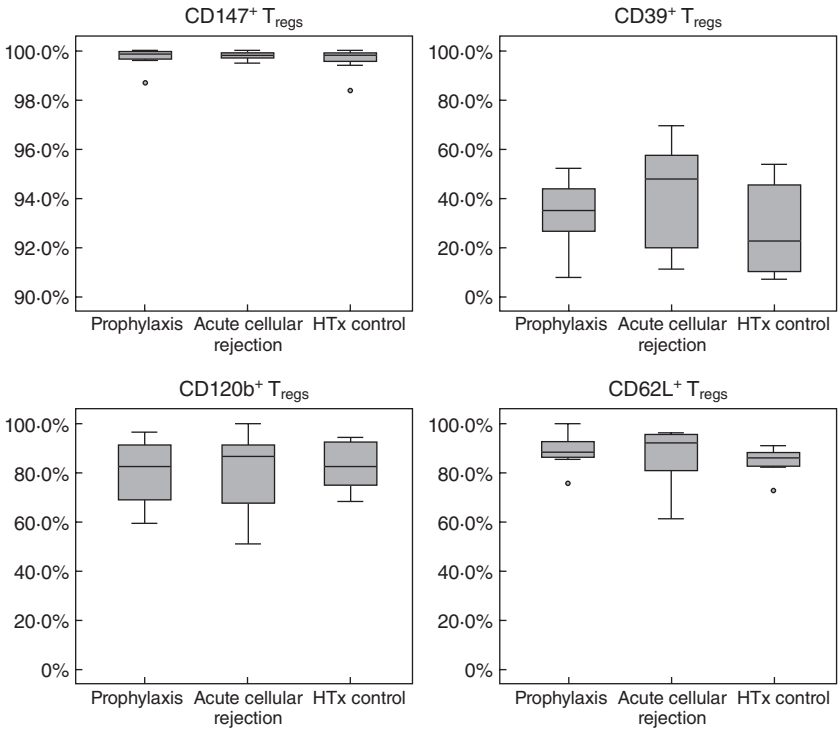


Fig. 3. Comparison of T cell (T_{reg}) subsets in heart transplantation (HTx) patients with prophylaxis or rejection as indication for extracorporeal photopheresis (ECP) and in HTx patients without ECP treatment. Different subsets of $CD3^+CD4^+CD25^{high}CD127^{low}$ T_{regs} were quantified regarding their expression of CD39, CD62L, CD120b and CD147.

To date, no monitoring strategy is available for ECP treatment after solid organ transplantation, and especially after HTx. Our study aimed to identify immunological changes of cellular parameters and of the cytokine profile, and to develop a monitoring strategy for ECP treatment.

Our data indicate that the immunological profile before ECP treatment may influence the effects of ECP treatment. Therefore, an immunological monitoring, especially before treatment, is necessary to estimate the therapeutic effects of ECP.

In our study, we detected changes in the percentage of the whole T_{reg} population, and for the first time analysed subsets of T_{regs} in ECP-treated patients and in the HTx control group. Previous studies on rodent animal models have already documented an increase of T_{regs} during ECP treatment [21,22]. Studies on lung transplant recipients reported increased levels for T_{regs} after ECP treatment [23,24]. Lamioni *et al.* [23] compared the percentages of T_{regs} in the blood of ECP-treated patients with healthy controls, but did not investigate the influence of the transplantation itself and the impact of immunosuppressive therapy on the immune system. Meloni *et al.* [24] found that the time–course of peripheral T_{regs} varied according to the graft function.

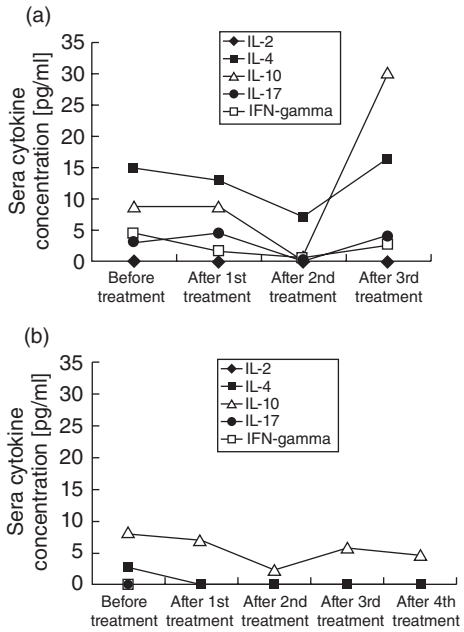
Table 2. Assessment of the immune balance in heart transplantation (HTx) recipients with prophylactic extracorporeal photopheresis (ECP) treatment (ECP prophylaxis group) and HTx recipients with acute cellular rejection as indication for ECP [acute cellular rejection (ACR) group].

	ECP prophylaxis group ($n = 9$)	ACR group ($n = 9$)
Increased Th1 cytokines	11% ($n = 1$)	22% ($n = 2$)
Increased Th2 cytokines	44% ($n = 4$)	0% ($n = 0$)
Increased Th1 and Th2 cytokines	33% ($n = 3$)	33% ($n = 3$)
Th1 and Th2 cytokine levels under LOD	11% ($n = 1$)	44% ($n = 4$)
Increased Th17 levels	22% ($n = 2$)	55% ($n = 5$)

LOD = limit of detection; Th1 = T helper type 1; Th2 = T helper type 2; Th17 = T helper type 17.

The high interindividual variability of the immune system may be responsible for weakened differences between the study groups. The quantified immunological effect of the ECP treatment displays a more eligible degree to evaluate the success of ECP treatment in a study cohort.

According to functional T_{reg} subset analysis, we recommend the enclosure of quantifying CD39-positive $CD4^+CD25^{high}CD127^{low}$ T_{regs} in a monitoring panel for ECP. CD39 marks activated effector/memory-like T_{regs} [17]. An increase in this cell population during ECP treatment argues for the induction of induced immunomodulatory processes, while it has been proposed that CD62L-, CD120b- and CD147-positive T_{regs} are not suitable for ECP monitoring at the current state of immunological research. Further studies are needed to investigate the functional details of T_{reg} subsets in immunomodulatory processes induced by ECP. Furthermore, research studies should investigate if T_{regs}, and especially their subsets with maximally suppressive potential, such as CD120b- and CD62L-



	Cumulative value Th1	Cumulative value Th2	Th17 value
Before treatment	4·53	23·89	3·01
After 1st treatment	1·67	21·71	4·75
After 2nd treatment	0·49	7·00	0·00
After 3rd treatment	2·70	46·55	4·15

	Cumulative value Th1	Cumulative value Th2	Th17 value
Before treatment	0·00	10·86	0·00
After 1st treatment	0·00	6·98	0·00
After 2nd treatment	0·00	2·13	0·00
After 3rd treatment	0·00	5·87	0·00
After 4th treatment	0·00	4·62	0·00

Fig. 4. One patient from the extracorporeal photopheresis (ECP) after rejection group (a) and one patient from the ECP prophylactic group (b) are shown as examples of the immune balance profile during the course of ECP therapy. Cumulative values for T helper type 1 (Th1) and Th2 cytokines as well as the Th17 values are shown.

positive T_{regs}, undergo a compartment change after ECP and may reside in one or more body cavities.

As well as T_{regs} and their subsets, DCs were analysed in our study because several studies have shown that DC subsets as well as immature DCs are linked to tolerance induction and maintenance [25–27]. In particular, pDCs support the expansion and induction of T_{regs} and are responsible for the maintenance of tolerance [28,29]. According to our results, we recommend the enclosure of pDC tracking into a monitoring panel for ECP.

Acquisition of the immune balance status is necessary for tracking immunological processes and for assessing the success of immune modulatory treatments. Based on our study results, we advise the acquisition and analysis of Th1, Th2 and Th17 cytokines for monitoring ECP treatment. The Th1 cytokines, IL-2 and IFN-γ, are proinflammatory cytokines while the Th2 cytokines, IL-4 and IL-10, promote anti-inflammatory activities of the immune system. An increase in cytokine levels after ECP that was reported in our study cohort can be attributed to the intervention itself and to the induction of immunological processes. Furthermore, the detected differences between the treatment groups in our study point to a different response to ECP and may suggest whether or not a rejection episode occurred before ECP treatment. Patients with prophylactic treatment tend to demonstrate a shift of the T cell responses in favour of Th2 cells after ECP. These data are in accordance with the documented effects of ECP treatment in other disease patterns, such as Sezary syndrome [30] or systemic sclerosis [31]. In contrast, patients with ACR as an indication for ECP showed a tendency for increased Th1 and Th17 cytokine levels during ECP treatment. IL-17 is produced by Th17 cells and is involved in inducing and mediating proinflammatory responses. Th1 cytokines show similar effects and support cellular immune responses. Thus, inflammation processes are favoured and chronification is supported. Additionally, patients with increased cytokine levels before ECP treatment showed a considerable reduction of cytokine levels after two complete ECP cycles. Possibly, these patients would benefit from a shortened ECP treatment.

According to our observations regarding the immune balance status of ECP-treated patients, we strongly recommend the documentation of patient-specific cytokine levels. Future studies should investigate the differences of prophylactic ECP treatments and ECP treatments with preceding ACR regarding the immunological response to ECP and the interactions of cell subsets after immunomodulatory stimuli. The influence of the immunosuppressive regimen should be included into the study design, and the outcome for clinical parameters (e.g. endomyocardial biopsy grading and the appearance of rejection episodes) as well as for laboratory parameters [e.g. changes in the human leucocyte antigen (HLA)-/non-HLA antibody status] should be evaluated in a larger study cohort. Further follow-up research projects should prove: (i) if the classification into patients with positive ECP effect / patients without ECP effect is clinically relevant, (ii) if the percentage of $CD39^+$ T_{regs} is a sufficient marker for monitoring ECP treatment and (iii) if the immune balance status is predictive for the success of ECP and transplantation outcome.

In summary, comprehensive analysis of subsets of T_{regs}, DCs and the immune balance showed that the monitoring of ECP treatment after HTx can provide additional information on the efficiency of ECP. This knowledge may be useful to shed more light into the immune modulation of

ECP therapy in HTx patients and, therefore, could be used to treat HTx patients with ECP more individually, e.g. (i) to define the starting-point of ECP therapy after transplantation or (ii) to define the duration and frequency of ECP therapy.

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Disclosures

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Author contributions

M. T. D. and M. J. B. conceived and designed the experiments; M. T. D. and A. P. performed the experiments; M. T. D. and A. P. analysed the data; H. B. B., S. D., F. W. M. and M. J. B. contributed reagents, materials and analysis tools; M. T. D. and M. J. B. wrote the paper; H. B. B., S. D., F. W. M. and M. J. B. supervised the study.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. One example of the flow cytometric gating strategy for the whole $CD3^+CD4^+CD25^{high}CD127^{low}$ regulatory T cell (T_{reg}) population is shown. Gated lymphocytes from the forward-scatter–side-scatter dot-plot were gated for $CD3^+$ $CD4^+$ cells. $CD3^+CD4^+$ T cells were analysed for $CD127^{low}$ and $CD25^{high}$ expression.
Fig. S2. One example of the flow cytometric measurement of $CD120b^-$ and $CD147^-$expression on $CD3^+CD4^+CD25^{high}$ $CD127^{low}$ regulatory T cells (T_{regs}) (panel 1) is shown. Samples that were stained with antibodies against CD3, CD4, CD25 and CD127 served as staining negative controls.
Fig. S3. One example of the flow cytometric measurement of CD39 and CD62L expression on $CD3^+CD4^+CD25^{high}$ $CD127^{low}$ regulatory T cells (T_{regs}) (panel 2) is shown. Samples that were stained with antibodies against CD3, CD4, CD25 and CD127 served as staining negative controls.